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Regulation and Activity of the Human *ABCA1* Gene in Transgenic Mice*

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The *ABCA1* transporter is one of the limiting steps in cellular cholesterol efflux. To study the expression and activity of the human *ABCA1* gene *in vivo* we have examined mice containing two human BAC transgenes with different 5' ends. Mice containing a 255-kilobase (kb) BAC transgene, including 70 kb upstream of the previously defined exon 1, demonstrated a pattern of tissue-specific expression mimicking that of the endogenous mouse gene. Compared with macrophages from control mice, macrophages from these transgenics had increases in apoA-I cholesterol efflux heightened in response to increases in cell cholesterol content. The observed increase in macrophage apoA-I-mediated cholesterol efflux was not accompanied by alterations in plasma high density lipoprotein in the transgenics. Although mice containing a smaller 171-kb human BAC transgene, lacking the previously described exon 1 and *ABCA1* promoter, did not express human *ABCA1* in macrophages, they did express the human transgene in liver at levels comparable with those of the orthologous mouse gene. Analysis by 5' rapid amplification of cDNA ends of liver mRNA from these animals revealed a new *ABCA1* exon 1 (exon 1A) and a previously unrecognized promoter. Analysis of human tissue revealed that exon 1A containing transcripts accounted for a high proportion of the *ABCA1* mRNAs present in human liver. This analysis of *ABCA1* transgenics showed that the expression of human *ABCA1* transgenes can result in increased cholesterol efflux from macrophages, unaccompanied by changes in plasma high density lipoprotein, and identified a new *ABCA1* promoter in humans.

A major mechanism postulated to contribute to the atheroprotective effects of high plasma HDL¹ is reverse cholesterol

transport. This is a process whereby HDL participates in the transfer of excess cholesterol from peripheral tissues to the liver for catalysis (1, 2). Specifically HDL is believed to protect against the accumulation of lipids in the vasculature by removing cholesterol and phospholipids through an active transport pathway (3, 4). This pathway is impaired in Tangier disease (5–7), a rare recessive disorder characterized by the accumulation of lipids in peripheral tissues, dramatic reductions in plasma HDL, and an increased risk for cardiovascular disease (8, 9). Recent studies have demonstrated that *ABCA1*, an ATP binding cassette transporter, plays a key role in apolipoprotein-mediated cholesterol transport (10) and that mutations in the *ABCA1* gene are the underlying genetic defect in Tangier disease (11–15). These results imply that the *ABCA1* transporter is a rate-limiting factor in reverse cholesterol transport and plays a key role in the protection against atherosclerosis.

Due to the importance of *ABCA1* in HDL formation and cholesterol efflux, great efforts have been made to define the genetic components and the cellular mediators determining *ABCA1* expression levels. Various *in vitro* (10, 16–18) and *in vivo* (19) studies of *ABCA1* expression have shown that this gene is tightly regulated by the cholesterol status of the cell. Studies have defined the human *ABCA1* promoter as spanning nucleotides –200 to –80 upstream of exon 1 (20). This promoter contains a DR-4 element that binds the LXR and RXR heterodimers and transactivates gene expression when bound to oxysterols and retinoic acid (21–23). This mechanism explains in part the tight regulation of the *ABCA1* gene by sterols and supports the idea that the response of *ABCA1* to increased cholesterol leads to an accelerated clearance of cholesterol by macrophages maintaining intracellular cholesterol homeostasis.

Although both mice and humans with reduced *ABCA1* have been characterized and shown to have reduced HDL levels and decreased cellular cholesterol efflux (8, 11, 24–27), the *in vivo* effect of increased expression of this gene and its regulation have not been characterized. Accordingly, we have examined transgenic mice with two different human *ABCA1*-containing BAC transgenes. The mice with the human *ABCA1* transgene with more human 5' DNA demonstrated largely appropriated regulation of the human *ABCA1* transgene. Surprisingly, the increased cholesterol efflux in macrophages was not accompanied by changes in plasma HDL levels. Analysis of the mice with a 5' truncated *ABCA1* transgene led to the discovery of a new *ABCA1* promoter and alternatively spliced transcript that is present at high levels in the livers of humans.

PCR, polymerase chain reaction; Abca1, mouse ATP binding cassette transporter 1; FBS, fetal bovine serum; LDL, low density lipoprotein; apoA-I, apolipoprotein A-I; RACE, rapid amplification of cDNA ends; LRH-1, liver receptor homologue-1.

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¹ The abbreviations used are: HDL, high density lipoprotein; *ABCA1*, ATP binding cassette transporter 1; kb, kilobase(s); LXR, liver X receptor; RXR, retinoid X receptor; BAC, bacterial artificial chromosomes;

TABLE I
Primers used in the TaqMan system and 5' RACE PCRs

Primer name	Sequence (5'-3')
Exon 1F	GGAGAAAGAGACGCAACACA
Exon 2R	AGCGGCCAGAGCTCACAG
Exon 1AF	TCCACAGTAGAACACAGTTGAACA
Exon 3F	CAACATGTCAGCTGTTACTGGAAG
Exon 4R	GAGCCTCCCCAGGAGTCG
Mouse exon 2F	CATTAAGGACATGCACAAGGTCC
Mouse exon 4R	CAGAAATCCTGCAGCTTCAATT

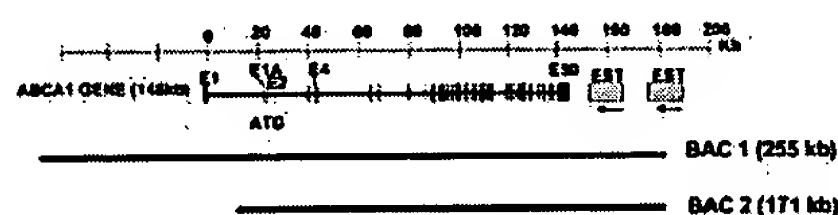


FIG. 1. Map of the human ABCA1 BACs. The schematic representation of the exon/intron structure in the human ABCA1 gene is shown at the top. The newly identified exon (exon 1A or E1A) is located 2,210 base pairs upstream of exon 2. The ATG initiation codon and the sequence encoding for the first 21 amino acids of the ABCA1 protein is located in exon 2. The expressed sequence tags represent the open reading frames of two identified expressed sequence tags with unassigned functions. BAC1 is 255 kb long, and it contains 70 kb upstream of exon 1 and ~35 kb downstream of the 3' untranslated region. The 171-kb BAC2 lacks exon 1 and its promoter but contains the new exon 1A and its promoter together with the complete coding sequence of ABCA1.

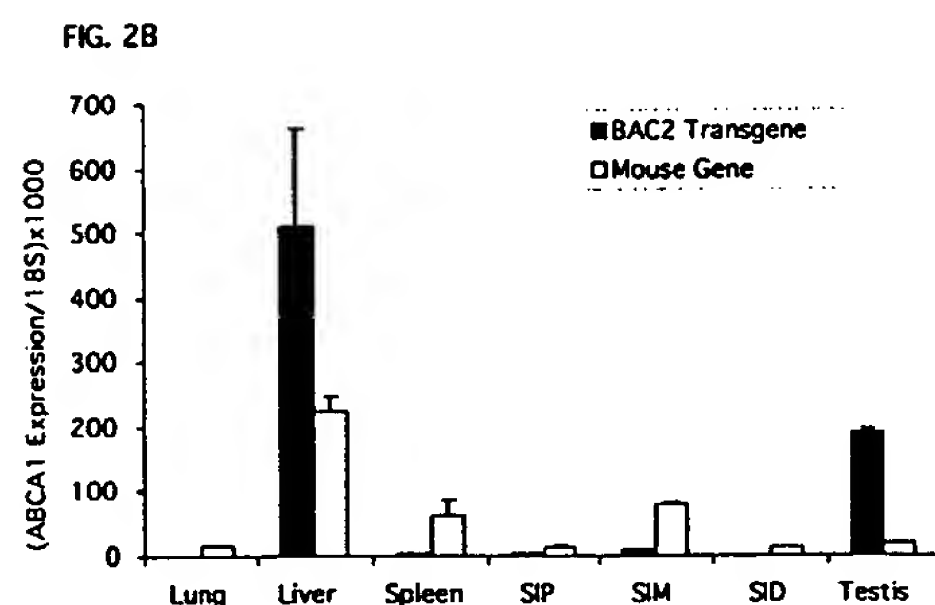
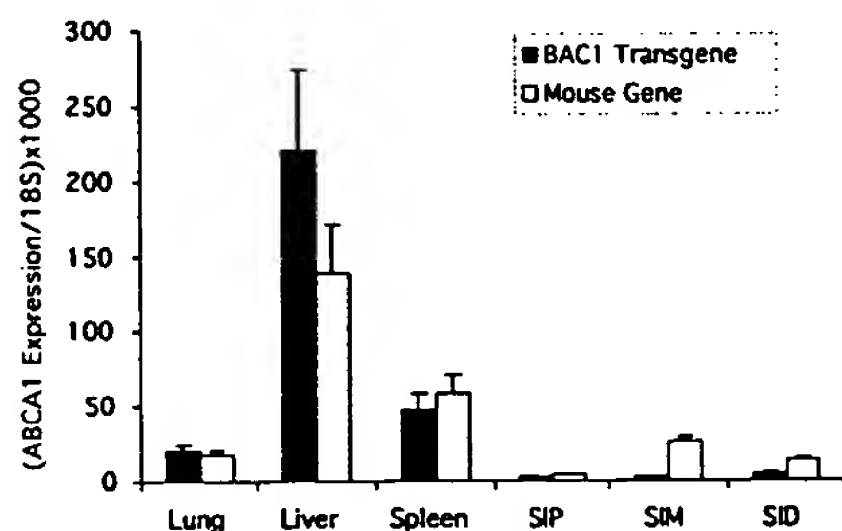


FIG. 2. The expression of human ABCA1 genes in transgenic mice. Total RNA was isolated from different tissues of a BAC1 (A) and a BAC2 (B) transgenic mouse. RNA levels of the ABCA1 genes were measured using a TaqMan quantitative assay using mouse Abca1 primers located in exons 2 and 4 and human ABCA1 primers in exons 3 and 4 (Table I). The relative transcript amounts were obtained by normalizing the mouse and human ABCA1 signals to the 18 S signals retrieved from the same tissues. The error bars indicate the S.D. of four TaqMan measurements. Similar results were obtained from at least two independent lines of BAC1 and BAC2 transgenic mice. SIP, SIM, and SID are abbreviations for the proximal, medial, and distal parts of the small intestine, respectively.

EXPERIMENTAL PROCEDURES

Mice—The ABC1 transgenics examined in this study were created and maintained in a pure FVB background as described previously (28).

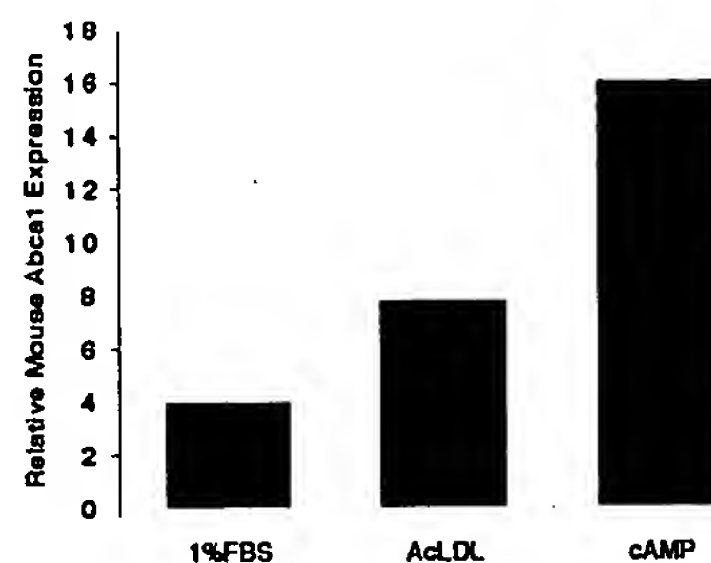


FIG. 3B

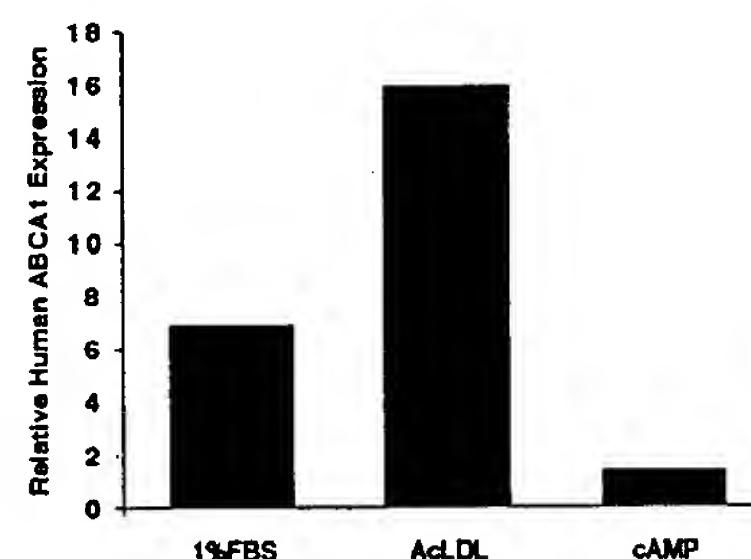


FIG. 3. The expression of mouse and human ABCA1 in peritoneal macrophages from BAC1 transgenics. Peritoneal macrophages isolated from five BAC1 transgenic mice were isolated as indicated under "Experimental Procedures." One million cells/well were plated in 24-well plates, allowed to attach to the plates for 4 h, and then washed with RPMI 1640 medium with 1% FBS. Two wells were incubated for 24 h with 1% FBS, 50 μ g of protein/ml of acetylated LDL (acLDL), or 0.3 mM 8-(4-chlorophenylthio)-adenosine-3':5'-cyclic monophosphate (cAMP). RNA levels of the ABCA1 genes were measured using a TaqMan quantitative assay using mouse Abca1 primers (A) located in exons 2 and 4 and human ABCA1 primers (B) located in exons 3 and 4. The relative transcript amounts were calculated by normalizing the mouse and human ABCA1 signals to the 18 S signal retrieved from the same RNA. Each column represents an average measurement of duplicate TaqMan experiments.

The structure of the two BAC transgenes analyzed in this study is depicted in Fig. 1. Both BAC1 and BAC2 encode the complete ABCA1 protein including the ATG methionine initiation site present in exon 2. The 255-kb BAC1 comprises the entire structural gene plus 70 kb of DNA upstream of the 5' initiation start site and at least 35 kb of DNA downstream of the polyadenylation signal. The 171-kb BAC2 lacks exon 1 and the 5' initiation site and contains 13 kb of DNA upstream of exon 2 and ~35 kb downstream of the polyadenylation signal. Studies of gene expression, cholesterol efflux, and plasma HDL were performed on at least two lines of BAC1 and BAC2 transgenic mice.

Diets and Plasma HDL Measurements—Mice were fed either Purina Mouse Chow (no. 5001) or a Western-type diet with 1.5 g/kg of cholesterol (Harlan Teklad, TD 88137) for 2 and 4 weeks. Blood samples were collected after an overnight fast, and HDL cholesterol was measured by standard methods described previously (29).

Expression Analysis of Human and Mouse ABCA1—Total murine RNAs from lung, liver, spleen, proximal, medial, and distal small intestine, and testis were extracted using the TRIZOL method (Life Technologies, Inc., catalog no. 15596). Total RNA from human tissues including liver, heart, lung, kidney, adrenal, intestine, brain, and testis was purchased from CLONTECH. Reverse transcriptions were performed using 5 μ g of total RNA, random hexamers (New England Biolabs), and Superscript II reverse transcriptase (Life Technologies, Inc.) at 42 $^{\circ}$ C for 60 min. The amount of cDNA generated was measured using the TaqMan Syber-green quantitative PCR assay with a 7700 ABI PRISM sequence detector system (PerkinElmer Life Sciences). The primer pairs used to detect the mouse endogenous Abca1 gene (mouse exons 2F and 4R), the human ABCA1 transgene (exons 3F and 4R), the human exon 1A transcript (exons 1AF and 2R), and the human exon 1 transcript (exons 1F and 2R) are listed in Table I. The relative amount of ABCA1 transcript was normalized to 18 S RNA amplification values

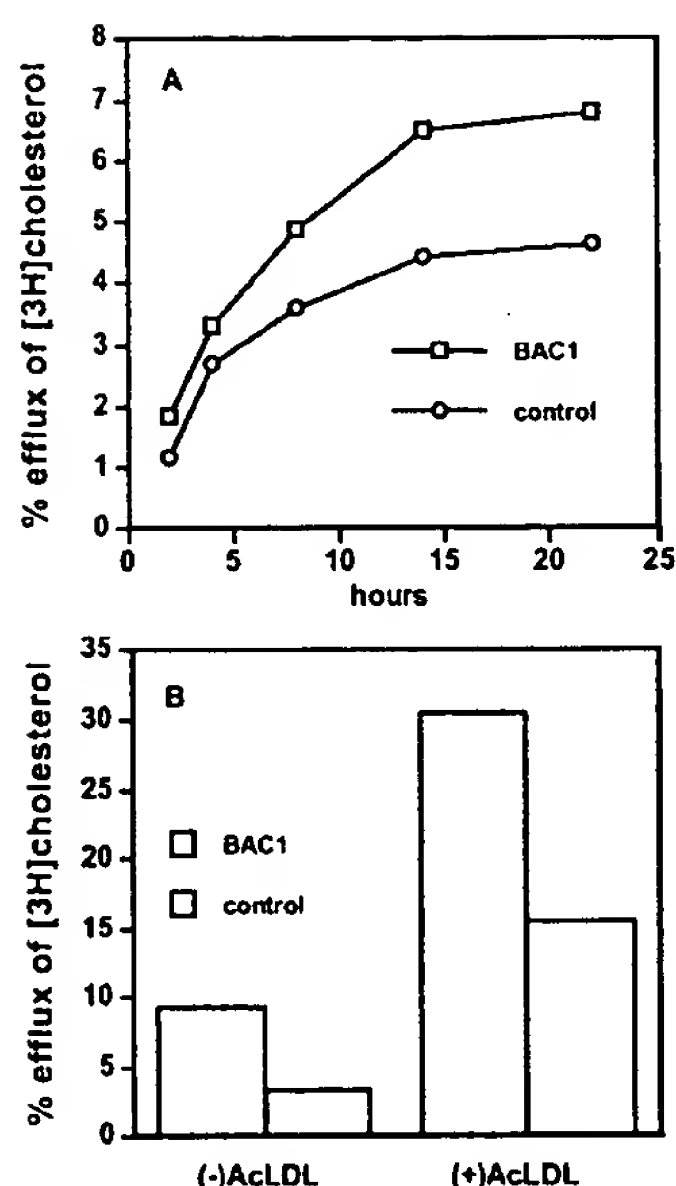


FIG. 4. ^3H -Cholesterol efflux in peritoneal macrophages from BAC1 transgenics. A, ApoA-I-mediated cholesterol efflux. Five BAC1 (\square) and five control mice (\circ) were used to isolate the peritoneal macrophages as described under "Experimental Procedures." The percentage of cholesterol was measured at the time intervals given after the addition of lipid-free human apoA-I. Each number represents an average measurement of two independent wells. B, ^3H -cholesterol efflux of cells incubated with or without acetylated LDL (AcLDL). Peritoneal macrophages were isolated from five BAC1 (gray columns) and five control mice (white columns). The efflux of cholesterol to apoA-I after 24 h was measured in the presence or absence of acetylated LDL. Each column represents an average measurement of two independent wells for each treatment.

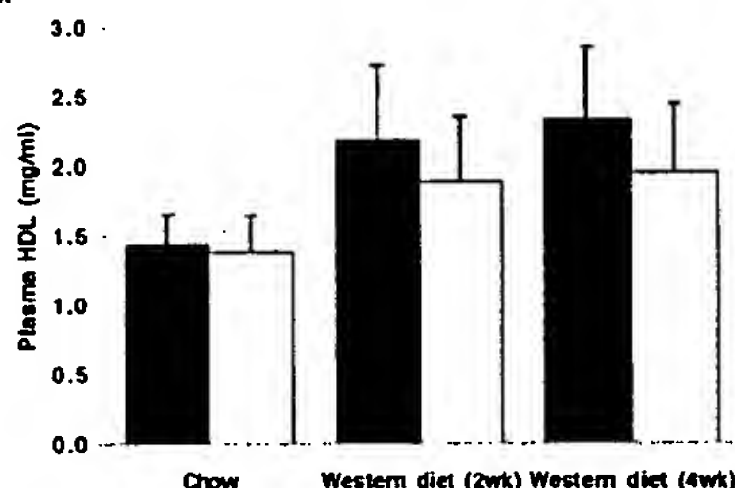


FIG. 5. Plasma HDL cholesterol in BAC1 transgenics. HDL cholesterol was measured in five 4-month-old BAC1 transgenic mice (\blacksquare , three male and two female) and five littermate control mice (\square , three male and two female) on Mouse Chow diet or Western diet for 2 and 4 weeks. Error bars indicate the S.D. of the five samples in each group.

retrieved from the same sample. The control 18 S RNA used to correct for the total RNA amounts in each sample was purchased from Ambion, and a ratio of 0.8:1.2 for primers/competimers was used for all the samples. PCR was performed with an initial hot start of 3 min at 94 °C followed by 40 cycles of 94 °C for 10 s, 57 °C for 15 s, and 72 °C for 30 s.

Isolation of Peritoneal Macrophages—Control and transgenic mice were injected intraperitoneally with 5 ml of thioglycolate solution. On the fifth day after injection, the elicited cells were isolated by peritoneal lavage with 5 ml of sterile phosphate-buffered saline. The cells were then pelleted by centrifugation for 10 min at 1,000 rpm and recovered in RPMI 1640 medium with 10% FBS. One million cells/well were plated on 24-well plates. The cells were allowed to attach to the plates for 4 h and were then washed with RPMI 1640 medium with 1% FBS.

Cholesterol Efflux—Peritoneal macrophages were labeled with 1 μCi /ml/well of ^3H -cholesterol (48 Ci/mmol) for 24 h. At the same time, cells were incubated with 0.3 mM 8-(4-chlorophenylthio)-adenosine-3':5'-cyclic monophosphate (cAMP), 50 μg of protein/ml of acetylated LDL, or

1% FBS. Prior to cholesterol efflux measurements, cells were equilibrated (30 min at 37 °C) with 0.2% bovine serum albumin in RPMI 1640 medium followed by brief rinses with serum-free RPMI. Cells were exposed to 25 $\mu\text{g}/\text{ml}$ lipid-free human apoA-I for 2, 4, 8, 14, and 22 h. Medium recovered from cells was centrifuged ($2,000 \times g$, 10 min) to remove cellular debris, and the supernatant was used to measure ^3H -cholesterol in the medium. Cell lipids were extracted using isopropanol, and efflux results were expressed as a percentage of the initial cellular ^3H appearing in the medium at each time point.

5' Rapid Amplification of cDNA Ends (RACE)—5' RACE was performed to determine the 5' end of the human ABCA1 transgene expressed in mouse liver. Total RNAs from the liver tissue of the BAC2 transgenic mouse were extracted using the TRIZOL method (Life Technologies, Inc.). First strand cDNA was generated from 1 μg of total RNA using Superscript reverse transcriptase (Life Technologies, Inc.) at 42 °C for 60 min. An aliquot of the first strand cDNA was then amplified using a human ABCA1-specific primer, exon 4R (Table I), and a universal primer (CLONTECH). PCR was performed with 40 cycles of 94 °C for 10 s, 57 °C for 15 s, and 72 °C for 30 s. The amplified products were analyzed by agarose gel electrophoresis and cloned into the TA cloning vector (Invitrogen) using conditions recommended by the manufacturer. Twenty-four single colonies were picked and sequenced from both ends using the ABI Prism BigDye terminator cycle sequencing kit (Applied Biosystems Division, Foster City, CA) with a 377 automated DNA-sequencing instrument (Applied Biosystems Division).

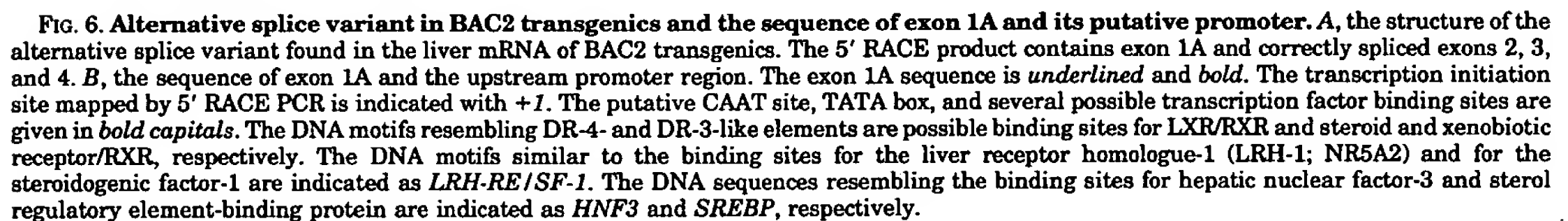
RESULTS

Expression of Human ABCA1 in Mouse Tissues—The tissue expression patterns of two human ABCA1 transgenes have been reported by Qiu *et al.* (28). We have examined several additional tissues (Fig. 2A), and the results support the theory that BAC1 contains the DNA necessary for appropriate tissue-specific expression of ABCA1. BAC2 contains the entire coding sequence of human ABCA1 and 13 kb upstream of exon 2 (Fig. 1). Although this transgene lacks the previously described promoter (20), it is expressed in liver and testis and at very low levels in all other tissues examined (Fig. 2B).

To determine how the transgene is regulated in macrophages, we measured the transgene expression changes in cholesterol-loaded and cAMP-treated mouse peritoneal macrophages. Both cholesterol and cAMP analogs induce the apolipoprotein-mediated cholesterol efflux and ABCA1 expression (10, 16–18, 30). The BAC1 transgene was expressed in peritoneal macrophages and induced by acetylated LDL in the same manner as the endogenous mouse gene (Fig. 3). The response to cAMP treatment was different for the human transgene and the endogenous mouse gene. The human transgene was down-regulated by the cAMP treatment, whereas the endogenous mouse gene was highly induced (Fig. 3). This may reflect differences in the kinetics of the response between the human and mouse ABCA1 genes in peritoneal macrophages. The response of ABCA1 to cAMP has previously been reported to differ between mouse and human cells, with mouse cells showing a high and immediate cAMP induction and human cells showing no induction or requiring a longer cAMP incubation time (17).

Cholesterol Efflux in Peritoneal Macrophages from BAC1 Transgenics—To address the question of whether the expression of the human transgene confers functional activity, we measured the efflux of cholesterol to apoA-I using peritoneal macrophages from BAC1 and control mice. Apolipoprotein-mediated efflux was ~2-fold greater in macrophages expressing the human ABCA1 transgene compared with controls (Fig. 4A). Macrophages from the transgenic and control mice responded similarly to acetylated LDL exposures with nearly 4-fold increases in efflux compared with the nonexposure cholesterol efflux levels (Fig. 4B). These findings are consistent with an up-regulation of ABCA1 gene expression with cholesterol loading of macrophages and demonstrate the human transgene mimicking the response of the endogenous mouse transporter.

Plasma HDL Cholesterol—The background for investigating



These receptors have been shown to mediate transcriptional regulation of many genes implicated in cholesterol homeostasis including the *ABCA1* gene (32). Although these DNA motifs have not been proven to interact with the transcription factors, they are likely targets for studying the regulatory function of ABCA1 in liver and testis.

Expression of the Exon 1A Transcripts in Human Tissues—In the BAC1 transgenics, both exon 1 and exon 1A transcripts are present in all tissues with the highest exon 1A expression level in the liver (data not shown). The observation that BAC1 transgenic mice express both exon 1 and exon 1A transcripts suggested that exon 1A may not be a cryptic start site. To determine whether the exon 1A transcripts are present in human tissues and to examine their potential physiological relevance, we investigated the expression of exons 1 and 1A in several human tissues (Fig. 7). Exon 1A was found to be very abundant in human liver, whereas the exon 1 transcripts were found to be more abundant in other peripheral tissues such as lung or adrenals. These results indicate that exon 1A occurs naturally in human tissues and is the predominant ABCA1 transcript in liver.

The expression pattern of the BAC1 transgene, including 70 kb of DNA upstream of exon 1, suggests that this human transgene likely contains many of the necessary sequences required for the proper regulation of ABCA1 *in vivo*. Although the observation that expression of this transgene was associated with increased apoA-I-mediated cholesterol efflux in peri-

Analysis of the Promoter Sequence—A comparison of the sequence of exon 1A with the genomic sequence identified a conserved 5' splice site located adjacent to this exon. Moreover, the sequence contains the classical TATA box and a CAAT site upstream of the initiation site, suggesting that it may be part of the promoter responsible for the transcriptional control of exon 1A (Fig. 6B). Transfac searches (31) and visual analysis of the promoter region have detected the presence of possible binding sites for several sterol-sensing nuclear receptors including LXRs, steroid and xenobiotic receptor, liver receptor homologue-1 (LRH-1), and hepatic nuclear factor-3 (Fig. 6B).

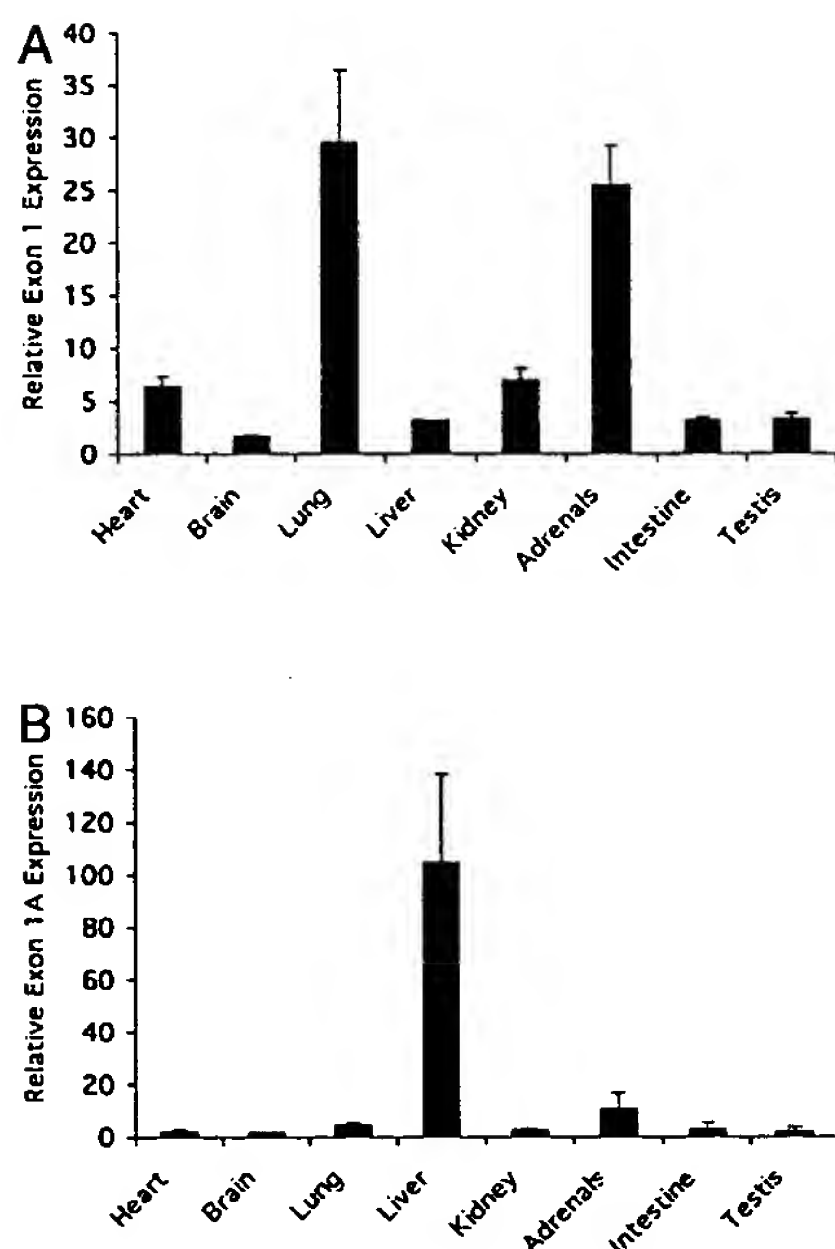


FIG. 7. The expression of exons 1 and 1A in human tissues. The relative expression of exon 1 (A) and exon 1A (B) transcripts was measured from the total RNA of different human tissues (CLONTECH) in a TaqMan PCR quantitative assay described under "Experimental Procedures." The signals for each tissue were corrected for the total amount of RNA given by the 18 S signal.

toneal macrophages was predicted, the finding that this was not associated with changes in plasma HDL on two different diets was surprising. An increase in plasma HDL was expected given the fact that both human and mice with decreased ABCA1 expression have decreased HDL values (8, 11, 24–27) and that up-regulation of the gene by rexinoids results in increased HDL plasma values (19).

One of the many possible explanations for the lack of changes in plasma HDL in the ABCA1 transgenics is that the increased efflux in these animals may be too low to impact plasma HDL. Induction of the ABCA1 gene by rexinoids, which results in enormous increases in ABCA1 expression in macrophages, is associated with only moderate increases in plasma HDL cholesterol levels (19). Another explanation for the absence of measurable increases in plasma HDL in BAC1 transgenics relates to the fact that plasma HDL only measures the steady-state level of this lipoprotein in plasma. The ABCA1 transgene may also affect HDL catabolism downstream of the efflux process, possibly in the liver, where it might compensate for the increased efflux occurring at peripheral tissue caused by increased ABCA1 expression. Finally, it can be hypothesized that there are other limiting parameters necessary for HDL formation such as apoA-I, which is not concomitantly up-regulated upon ABCA1 over-expression. Through the analysis of higher expressing ABCA1 transgenics and the crossing of ABCA1 and apoA-I transgenics coupled with more in-depth metabolic studies should help elucidate the relationship between increased expression of ABCA1 and plasma HDL levels.

A surprising result of this study was the expression of the smaller human BAC2 transgene in liver and testis despite the absence of the previously defined ABCA1 promoter (20). The 5' RACE analysis of liver RNA from animals expressing this transgene identified a new ABCA1 exon (exon 1A) preceded by a sequence containing the hallmarks of a classical promoter.

This new transcriptional start giving rise to a properly spliced alternative variant was noted to be the predominant ABCA1 form in human liver, supporting the physiological relevance of this transcript. Other alternatively spliced variants have been detected previously in HepG2 cells (21), but they do not correspond to the exon 1A variant identified in the present study; and although they change the amino acid sequence of the protein, their function has not been further characterized. The ABCA1 liver variant described in the present study has a different 5' untranslated region but no alterations in its protein sequence. This variant ABCA1 may confer a new level of regulation to the gene either at the transcriptional level, through the use of an alternate promoter, or the translational level, with the new 5' untranslated region possibly affecting the intracellular site and efficiency of ABCA1 translation.

Several features of the newly identified exon 1A transcription variant suggest that it may contribute to tissue-specific control of ABCA1 expression. This includes the detection of possible LXR/RXR and LRH-1/steroidogenic factor-1 binding sites at the 5'-flanking region of exon 1A. LXRs are ligand-activated transcription factors that mediate the regulation of several genes implicated in cholesterol homeostasis (32, 33). LRH-1 is a monomeric nuclear receptor that functions as a tissue-specific transcription factor and acts as a competence factor mediating the liver-specific regulation of the cholesterol 7 α -hydroxylase gene by LXR α (34–36). Steroidogenic factor-1 has DNA binding requirements similar to LRH-1 and controls the oxysterol-mediated transactivation of genes involved in steroidogenesis and gonad development (37). The presence of these DNA motifs in the 5'-flanking region of exon 1A can potentially account for the expression of BAC2 in liver and testis. The putative exon 1A promoter might allow for an alternate regulation of ABCA1 by liver-specific by-products of cholesterol catabolism and in that way contribute to the coordinate regulation of ABCA1 in the different steps of reverse cholesterol transport.

Modification of HDL metabolism has long been considered a target for therapeutic interventions in the prevention of atherogenesis (for a recent review, see Ref. 38). With the discovery of ABCA1 and its impact on HDL metabolism effecting cholesterol efflux and intestinal absorption of dietary cholesterol (17, 19, 27), ABCA1 has become a major target for intervention. Because of the expression of ABCA1 in many tissues and its multiple apparent functions (25, 39–41), only through *in vivo* studies can we begin to assess the relevant physiological effects of increasing its expression on lipid homeostasis and atherogenesis. The results of the present analysis of the *in vivo* effects of the human ABCA1 transgene illustrate the complexity of ABCA1 regulation and its impact on HDL formation and metabolism. Analysis of the atherogenesis susceptibility of these BAC transgenics with increases in cholesterol efflux unaccompanied by measurable changes in plasma HDL cholesterol should provide important insights into the utility of altering the expression of ABCA1 expression on atherogenesis.

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